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(57) Abstract

A new isolated protein is described having no O-glyclosylation and at least 70 % homology of amino acid sequence with human serum CD14. In addition, a composition comprising an effective amount of the protein and use of a CD14 variant in the treatment or prevention of a disorder of the gastro-intestinal tract of a mammal are described. In particular the disorder is selected from the group which comprises inflammatory bowel disease, chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonatal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

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Protein For Treatment Or Prevention of A GI Tract Disorder

The present invention relates to a new isolated protein from mature human milk; a composition comprising it; a method for manufacture of the protein or the composition; use of the protein, a variant or fragment thereof in the manufacture of a medicament or nutritional product for the treatment or a gastro-intestinal (hereinafter GI) tract disorder; and a method of treatment or prevention of the disorder which comprises administering an effective amount of the protein or composition..

Within the context of this specification the word “comprises” is taken to mean “includes, among other things”. It is not intended to be construed as “consists of only”.

Within the context of this specification, the terms AMP, CMP, GMP and UMP are taken to mean respectively the monophosphates of adenosine, cytidine, guanosine and uridine and their nucleotide equivalents which include polymeric RNA, ribo-nucleosides, ribonucleoside containing adducts and di-and triphosphate ribo-nucleotides.

It is well known that infant formulae are generally designed to resemble human milk as closely as possible. However, a plurality of constituents in human milk are bioactive and, because of synergies among the constituents, the inclusion of just one or a few of them in the infant formula may not produce the bioactivity observed in human milk.

In addition, bioactivity of the constituents may be affected by heat treatment for sterilisation and long term storage of the formula.

These problems are compounded in view of the fact that not all of the constituents have been identified and there are variations in the concentration of components which are present, possibly due to variations of mother’s diets. Therefore there are difficulties in formulating infant formulae which resemble human milk.

It is known that pharmaceutical compounds have wide application and some may be used in the treatment of patients suffering from disorders of the GI tract. However, a number of the known compounds are not naturally occurring and in view of this, patients may be reluctant to be administered them. In the light of this there is a need for the provision of new products which include naturally occurring compounds that have a nutritional or therapeutic effect.

A problem with some commercially available products is that they give rise to side effects such as nausea, bloating, cramping, allergy etc. Clearly there is a need for a product which does not give rise to these side effects.

The method of administration of a nutritional or therapeutic compound is an important consideration. Intravenous or subcutaneous administration requires expertise and compared to oral administration it is not as safe, convenient or acceptable to the patient. In the light of these concerns it is clear that there is a need for new nutritional or therapeutic products which can be administered orally.

The protein, CD14, is a myeloid cell-surface glycoprotein which acts as a receptor for bacterial lipopolysaccharide. It is well documented that monocyte/ macrophage activation by lipopolysaccharides via membrane CD14 (mCD14) triggers the release of a variety of pro-inflammatory, immunoregulatory and cytotoxic molecules such as TNF-α, IL-1, IL-6, IL-8, oxygen radical products and nitric oxide. mCD14 lacks transmembrane and cytoplasmic domains. It is anchored to the cell membrane by a glycosyl-phosphatidylinositol linkage.

In addition to the membrane bound form, soluble CD14 (sCD14) has been identified in normal human blood serum, hereinafter referred to as serum sCD14. Serum sCD14 exists in two forms, serum sCD14α (49 kDa) and serum sCD14β (55 kDa). It has been demonstrated that serum sCD14 binds lipopolysaccharides and mediates the lipopolysaccharide–induced activation of cells that lack mCD14, including epithelial and endothelial cells and astrocytes, as well as mCD14 expressing cells, such as monocytes and neutrophils.

The main source of serum sCD14 in normal human plasma is the monocyte. Monocytes release the two isoforms of serum sCD14, α and β, into plasma. The

former is produced by limited proteolysis from membrane-bound CD14 and the latter is directly derived from the intracellular compartment. The sCD14β:sCD14α ratio in culture supernatant of normal monocytes is approximately 2:1. However, in plasma from normal donors the serum sCD14α levels are either similar to, or even higher than, those of serum sCD14β, suggesting that the amount of serum sCD14β released *in vivo* is either lower or other cell types may contribute to the plasma pool.

A substantial concentration of serum sCD14 is found in normal human plasma, 2-3 μg/ml. In sera of septic patients global concentrations of serum sCD14 are elevated, reaching around 4 μg/ml. It has been reported a correlation between high levels of serum sCD14 at the onset of septic shock and poor outcome in septic patients.

WO98/22580 discloses the presence of a protein in bovine colostral why which has some amino acid sequence similarity with human serum sCD14. It is speculated that this protein could be the bovine variant of CD14. Figure 7 of the document shows the differences between the amino acid sequences of bovine colostral whey CD14, and the sequences of human serum CD14 and mouse serum CD14 which were known in the literature. In addition, the document describes affinity purification of a CD14 protein from human colostrum using a sepharose column having the monoclonal antibody 63D3 bound to it. However, the document does not describe an isolated CD14 variant purified from mature milk. Instead, colostrum is disclosed as the source of the protein. This is early milk from about the first one or two days post partum. It is yellow in comparison to the whiter colour of mature milk, it comprises a higher lipid content than mature milk and has a different nutritional value. It is well known that the mammary gland of first few days post partum undergoes a process of "physiological inflammation" and a large number of inflammatory cells such as neutrophils are present. This process allows leakage of serum factors including serum CD14 into the colostrum. Crucially, in view of this, the disclosure of WO98/22580 is considered to disclose purification of serum sCD14 or a complex mixture of proteins including serum CD14 from colostrum.

Remarkably, a new variant of CD14 has now been identified. It has been isolated from mature human milk. Suprisingly, this new variant is not the same

as serum CD14. Indeed, as described below, it differs from the known CD14 variants including the variants disclosed by WO98/22580 at least insofar as the new protein comprises no O- glycosylation. In stark contrast, the known CD14 variants including human serum sCD14 have both O- and N- glycosylation. Furthermore, it has now been found that CD14, a variant or fragment thereof retaining the bioactivity of CD14, or a composition comprising it is effective in the treatment of GI tract disorders.

Accordingly, in a first aspect, the invention provides an isolated protein having no O-glyclosylation and at least 70% homology of amino acid sequence with human serum CD14.

In a second aspect, the invention provides a method of production of the protein which comprises isolating it from mature milk.

In a third aspect, the invention provides a composition which comprises the protein excluding mature milk.

In a forth aspect, the invention provides a method of production of the composition which comprises adding an embodiment of the protein according to the invention.

In a fifth aspect the invention provides use of a CD14 variant or fragment that retains the bioactivity of CD14 in the manufacture of a nutritional product or medicament for the treatment or prevention of a GI tract disorder.

In a sixth aspect the invention provides a method of treatment or prevention of a GI tract disorder which comprises administering an effective amount of a CD14 variant or fragment thereof which retains the bioactivity of CD14.

The new protein has the unique capacity of being capable of mediating bacterial interaction with intestinal surfaces. Furthermore, if the new variant is included in an infant formula, the formula closely resembles mature human milk in its protective capacity of the intestinal surface.

Preferably, the amino acid sequence of an embodiment of the protein is at least about 90% homologous with the amino acid sequence of human serum CD14, more preferably at least about 95% homologous, even more preferably it is substantially identical. In alternative embodiments the amino acid sequence preferably has these degrees of homology with the amino acid sequence of bovine or buffalo CD14.

Preferably, an embodiment of the protein has at least one N- glyclosylation site. Preferably it has a plurality of N- glycosylation sites, more preferably from about 1 to about 10. Even more preferably, an embodiment of the protein has from about 3 to about 5 N- glyclosylation sites, most preferably 4.

Preferably, the presence of an embodiment of the protein is not revealed in a Western blot by the known commercially available anti-CD14 monoclonal antibody MY4.

Preferably an embodiment of the protein is isolated from mature milk. More preferably it is isolated from mature human, bovine or buffalo milk. Most preferably the protein is isolated mature milk sCD14 (hereinafter referred to as mmsCD14). In alternative embodiments it is produced recombinantly by standard techniques.

Preferably an embodiment of the composition comprises an embodiment of the protein together with a physiologically acceptable carrier, adjuvant or diluent. More preferably it comprises a compound extracted from milk, even more preferably a plurality of compounds extracted from milk. Most preferably the composition is an infant formula or enteral composition.

Preferably an embodiment of the composition comprises a casein fraction and milk fat. These two components of milk based products provide the advantage that they can preserve molecules from the proteolytic activity of the digestive tract. The biological activity of the protein according to the invention takes place in the small intestine after the passage through the gastric environment.

Preferably an embodiment of the composition comprises a lipopolysaccharide binding protein (LBP), decay accelerating factor (DAF, CD55), bactericidal

permeability increasing factor (BPI) or a mixture thereof. The advantage provided by these molecules with a protein according to the invention is that they participate in molecular recognition of the bacterial products and/or complement its defensive function.

The invention will now be described with reference to the accompanying drawings in which:

Figure 1A illustrates a comparative SDS-PAGE pattern of sCD14 of mature human milk (several dilutions (from 1:6 to 1:100) and in normal human plasma serum (NP, 1:50), with a rabbit polyclonal antibody.

Figure 1B illustrates the lack of milk CD14 detection by an antibody specific for the C-terminus peptide of the β serum CD14 molecule.

Figure 2 shows interleukine-8 release by undifferentiated HT29 cells following a 24h incubation with 100 ng/ml of *E. Coli* LPS in the presence of either 10% human serum (HS, pooled serum AB^{+}) or 1.7% human milk (HM, pooled mature human milk). To verify the role of sCD14 in induction, 20 μg/ml of monoclonal anti-CD14 antibody (MY4) or isotype control (IgG2b) was added to serum or milk before stimulation with LPS.

Figure 3 shows interleukine-8 release by undifferentiated HT29 cells following a 24h incubation with 2.5×10^{6} *E. Coli* in the presence of either 10% human serum (HS, pooled serum AB^{+}) or 1.7% mature human milk (HM, pooled breast milk). The role of sCD14 was confirmed by the inhibition of the IL-8 production when 20 μg/ml of monoclonal anti-CD14 antibody (MY4) was added to serum or milk before stimulation with LPS.

This invention is based upon the finding that mature milk comprises an unknown variant of CD14. The new molecule has a high homology at the amino acid sequence with the serum sCD14 molecule, however it has a different gel mobility that either of the two serum soluble forms, is the secretory product of the mammary gland epithelial cell, has a different glycosylation pattern with regard to the known forms, and has a unique capacity for mediating bacterial interaction with intestinal surfaces.

Furthermore, the invention relates to the use of the new form of soluble CD14 molecule, present in human, bovine and buffalo milk, in infant formula, clinical nutrition and animal feed.

In contrast with serum sCD14, where the α and β forms have been described, in human milk only a single major species can be detected. It differs in its tissue of origin -it is produced by the mammary gland epithelial cells- and the electrophoretic mobility.

Human serum and milk samples were analysed by SDS-PAGE under reducing conditions (Phastsystem®, Pharmacia) followed by western blotting with rabbit polyclonal anti CD14 antibody (Ab) and detection by enhanced chemiluminescence method (Amersham). The anti-CD14 monoclonal Ab, MY4, extensively used in this field for the detection of membrane-bound and soluble serum CD14 –both α and β- , failed to detect sCD14 in human milk.

The amino acid sequence including the N- and C-terminus ends of the mature human milk derived molecule is substantially identical to that of serum CD14 showing that milk and serum CD14 are highly homologous.

N- and C-terminal sequence analysis shows that milk CD14 is not posttranslationally truncated and corresponds therefore to the β form found in serum. But, whereas the β-form has now been found to migrate on SDS-PAGE with about 55KDa, milk CD14 migrates on SDS-PAGE only with about 48 KDa. This difference indicates that mature human milk and serum CD14 have different glycosylation patterns. Indeed, whereas CD14 has been reported to be N- and O-glycosylated, deglycosylation assays with milk CD14 showed no O-glycosylation. The presence of only N-glycosylation was confirmed by LC-MS analysis of N-glycosidase F treated mmsCD14 in that the resulting molecular mass corresponded to the theoretical molecular mass based on the published amino acid sequence. The main form of mature human milk sCD14 is therefore different from blood sCD14 that was reported to be N- and O-glycosylated.

An optical biosensor assay (Iasys, Affinity Sensors, Cambridge, UK) which implements advanced resonant mirror optical biosensor technology was utilised

to study the specific interaction of human serum CD14 and mature human milk CD14 with the mAb MEM-18 (Biogenesis, Poole, UK).

The MEM-18 mAb and a control isotype antibody (mouse IgG1, BectonDickinson) were immobilised in 10 mM Na-acetate at pH 5 on a dual well carboxymethyl dextran matrix of an IAsys cuvette using standard EDC [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide-HCl] and NHS (*N*-hydroxysuccinimide) coupling chemistry and subsequent blocking with ethanolamine. Running buffer was PBS/Tween 20.

Human serum (diluted 1:5 in PBS/Tween 20) and mature human milk (diluted 1:30 in PBS/Tween 20) samples (5 μL) were pipetted into the cuvette wells and their respective binding to both the specific MEM-18 and control surface were logged with time. It was observed that the mature human milk sample yielded an approximate 10-fold decrease in biosensor response within the first 2-3 minutes of protein-antibody interaction compared to that of the human serum sample. This indicates that the milk-borne CD14 has different/slower binding kinetics to MEM-18 than the serum-derived CD14.

The effect of milk-borne sCD14 on LPS stimulation of the mucosal surface, specifically the epithelial layer, was studied with IEC models *in vitro*. More specifically the IEC production of the cytokines IL-8 and TNF-α following endotoxin challenge has now been analysed. Three different human IEC lines, HT-29, SW620 and Caco-2 were used.

It is known that lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide binding protein and serum sCD14. LPS induces IL-8 secretion by IEC in the presence of human serum. This effect is maximum at an LPS concentration of 100 ng/ml. mmsCD14 has now been tested for its capacity to mediate LPS effects on IEC. Different mature human milk concentrations were assayed in IEC serum-free culture media containing a fixed dose of LPS (100 ng/ml). IL- 8 production increased with increasing concentrations of human milk (0-10%).

The contribution of mmsCD14 to stimulation of HT-29 cells by gram-negative non-pathogenic *Escherichia coli* has now been tested. An increasing quantity of

IL-8 secretion has been observed with increasing concentrations of bacteria in the range of 1 X10^3, to 5 X10^6 CFU per ml in the presence of 2% human milk. Furthermore, stimulation with 10^6 E. coli/ml of cell culture in the presence of 2% human milk induced ENA-78. Furthermore stimulation of intestinal epithelial cells with LPS and E. coli in the presence of milk-derived sCD14 induced expression of the β-defensin , HBD-2, that participates in the protection of mucosal surfaces against bacteria. Furthermore, mmsCD14 is more efficient than serum sCD14 in its stimulatory function of intestinal epithelial cells by bacteria or lipopolysaccharide.

An embodiment of the enteral composition of the invention preferably comprises an effective amount of mmsCD14, and preferably contains at least about 25 μg/ml of mmsCD14. The other components of the enteral composition are those conventionally added to infant formulae or enteral products and may be at least one of those described below.

Preferably the mmsCD14 may be any form of mmsCD14, but is preferably the form found in mature human milk and recognised by a polyclonal rabbit antibody. This form has a molecular mass of approximately 48 KDa, an electrophoretic mobility faster than the serum alpha form and cannot be recognised by the commercially available anti-CD14 antibody MY4. Alternatively the mmsCD14 may be extracted from bovine, buffalo, goat or sheep milk. In addition, it can be produced by recombinant microorganisms; for example recombinant fungi or yeast.

The supplementation of baby formula with this mmsCD14 has physiological benefit during the baby's neonatal period. This period is characterised by bacterial challenge due to bacterial colonisation and antigen challenge that could lead to inflammation, septic conditions or an allergic reaction.

The invention includes the use of a CD14 variant or fragment thereof having CD14 activity of mediating interaction of bacteria with an intestinal surface in the manufacture of a composition for the treatment or prevention of a disorder of the gastro-intestinal tract of a mammal. In particular the disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic

hepatitis, necrotising enterocolitis, neonotal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body. Mammals most likely to develop these disorders are premature, mal-nourished, immuno-depressed or mammals under trauma conditions.

Preferably the CD14 variant or fragment thereof is an embodiment of the protein according to the invention.

An embodiment of a composition according to the invention preferably comprises about 1.8 to about 4.5 g protein/100 kcal, preferably about 1.8 to about 3.6 g/100 kcal. The protein may be any suitable protein such as cow's milk protein, casein, whey, soy protein, egg protein, pea protein or a mixture thereof. The protein may be in the form of a salt, such as a caseinate. It may be an isolate or concentrate. Furthermore, the protein may be in intact or hydrolysed form. Alternatively or in addition, free amino acids may be used. Preferably a mixture of the whey : casein mass ratio is 60:40.

Whey protein can be prepared to have reduced allergenicity using conventional techniques such as described in U.S. Patent 5039532. For example, it can be prepared by electrodialysis or ultrafiltration.

An embodiment of a composition according to the invention preferably comprises about 7g to about 14g/100kcal of carbohydrate or provide 40 to about 60% of calories as carbohydrate. The carbohydrate can be supplied in a conventional form including simple form or complex form. Simple carbohydrates include lactose, maltose, sucrose and corn syrup solids. Complex carbohydrates include starches and maltodextrins. Starch may be precooked or pregelitanised.

An embodiment of a composition according to the invention preferably comprises about 3.3 to 6.5 g/100 kcal of fat. The fat can be supplied in a suitable form including saturated fat, monounsurated fat (MUFA), polyunsaturated fat (PUFA) or a mixture thereof. Preferably, fat is provided as about 1/3 saturated fat, about 1/3 MUFA and about 1/3 PUFA. Saturated fat includes butyric, valeric, caproic, capylic, decanoic, lauric, myristic, palmitic, steraic, arachidic, behenic and lignoceric fatty acids. MUFAs include palmitoleic, oleic, elaidic, vaccenic

and erucic fatty acids. Preferred PUFAs are C18, C20 or C22 ω-3 or C18, C20 or C22 ω-6 polyunsaturated fatty acids. Most preferred are the C20 or the C22 ω-3 or C20 or C22 ω-6 polyunsaturated fatty acids. These include not only arachidonic acid (ARA) but also eicosapentaenoic acid (EPA) and docoshexaenoic acid (DHA).

More than one PUFA can be added. In this case, two or more PUFAs may be from a different source, they can be added either separately or together during preparation of the composition according to the invention. For example, fish oil containing DHA may be mixed with one or more microbial oils containing another PUFA (e.g. ARA).

An embodiment of a composition according to the invention preferably comprises a carbohydrate : lipid weight ratio which is greater than 60:40. Preferably the ratio of carbohydrate to lipid is between 65:35 and 90:10. More preferably still it is between 70:30 and 85:15.

An embodiment of a composition according to the invention preferably comprises a nutritionally acceptable quantity of a minerals or vitamin selected from the group which comprises calcium, phosphorus, potassium, sodium, chloride, magnesium, iron, copper, zinc, manganese, iodine, selenium, vitamin A, vitamin D, vitamin E, vitamin K1, vitamin B1, vitamin B2, vitamin B6, vitamin B12, vitamin C, pantothenic acid, niacin, folic acid, biotin, choline, inositol or a mixture thereof.

An embodiment of a composition according to the invention preferably comprises an additional nutritional factor selected from taurine, carnitine, etc. The composition may contain at least about 7.5 mmoles carnitine /100 kcal.

An embodiment of a composition according to the invention preferably comprises one or more of AMP, CMP, GMP or UMP.

An embodiment of a composition according to the invention preferably comprises a pharmaceutically acceptable filler, color additive, taste modifier, non-antagonistic antibiotic, pharmaceutical, medicament or mixture thereof.

An embodiment of a composition according to the invention is preferably in a form suitable for ingestion orally or for administration by enteral tube feeding. More preferably it is an oral formulation.

A composition of the present invention is preferably formulated to provide about 600 to about 800 kcals/litre, more preferably about 670 kcals/litre. The total volume per dose is about 50 top about 200 ml preferably about 100 to about 150 ml.

The composition is preferably a solid, in which case it is preferably dried, and optimally in the form of a powder. Preferably, it is miscible or dispersible in an aqueous liquid, such as water.

Alternatively the composition is a liquid which is ready for use, or a concentrated liquid which can be diluted before use, preferably with water.

An embodiment of a composition according to the invention is preferably prepared by a method which comprises the following steps; (1) Standardising pasteurised milk (skimmed, evaporated or whole milk) by the addition of whey protein concentrate, minerals, water-soluble vitamins, trace elements and carbohydrates at a high temperature, for example 60°C, (2) mixing vegetable oil, oil-soluble emulsifier, at least one oil-soluble vitamin and antioxidant at high temperature, for example 60°C, (3) adding the oil mixture obtained from (2) to the standardised milk obtained from (1) with sufficient agitation to allow mixing, (4) homogenising the mixture obtained in (3) in two stages at high temperature and pressure, for example 60°C at 150 and then 30 bar, (5) cooling the emulsion obtained under (4) to a low temperature, for example 5°C, (6) adding water-soluble vitamins, minerals and trace elements to the cooled emulsion if desired, (7) sterilising the emulsion obtained under (6) on-line at ultra high temperature (UHT) and/or in an appropriate container to obtain a formula in the form of a sterile liquid or pasteurising and spray drying the emulsion (6) to give a spray dried powder which is filled into appropriate containers and (8), if desired, adding other dry ingredients, e.g. vitamins, minerals, trace elements, whey protein concentrate and carbohydrates to the spray dried powder by dry mixing.

The enteral composition may be supplemented with an embodiment of the protein according to the invention by adding it to the oil during processing. This provides the advantage that a production plant, or the process need not be significantly modified. However, adding the protein at such an early stage can have disadvantages because it may be degraded. In the light of this, alternatively it is added at a later stage. This provides the advantage that exposure of the protein to unfavourable conditions is minimised. Preferably, the protein is added after drying.

The protein can be added in a variety of forms. It may be added in solution, for example in a lipid and/or an oil composition. The oil may contain solely the protein or it may contain a number of other ingredients. If a solid composition is used, the protein may be encapsulated in capsules or it maybe in a powdered form.

In an embodiment of a process according to invention it is preferred that the starting oil phase does not contain any PUFAs. Preferably they are added later, preferably after drying.

The enteral composition and the use of this enteral composition according to the invention are described in further detail in the tests and examples described below where percentages are given by weight, except where otherwise indicated.

<u>Test 1:</u> Ion exchange chromatography for purification of mmsCD14 from mature milk (bovine, buffalo, goat or sheep) and supernatant of CD14 cDNA transfected cells.

The purification of mmsCD14 from milk or culture supernatant of transfectant cells was performed by ion exchange chromatography. Diluted milk samples or conditioned medium of CD14 cDNA transfectant cells were applied to a Mono Q10/10 column equilibrated with 20mM ethanolamine pH 9.5 . After washing, the column was subjected to a linear gradient of NaCl (0-500mM) in the equilibrating buffer. Fractions are collected and the mmsCD14 content was determined by ELISA.

Further characterisation by reverse phase HPLC was carried out to confirm the purity of the protein. The selected fractions were pooled , desalted with PBS, and kept aliquoted at –70°C until further use. The purified material was routinely characterised by N-terminal sequencing, amino acid analysis, SDS-Page, and mass spectrometry.

Test 2: Detection of mmsCD14.

In vitro stimulation studies of intestinal epithelial cell lines (HT29 and SW620) with several dose of LPS (Sigma O55B5) or with *E. Coli* bacteria in the presence and/or absence of either mature human, buffalo or bovine milk, and anti-CD14 antibodies were carried out.

The human intestinal epithelial cells HT29 (CD14-negative) were stimulated with varying concentrations of LPS (0.1 to 1000ng/ml) in the presence or absence of human AB serum (10%). After 24h culture at 37°C, culture supernatants were collected and tested for IL-8 release by ELISA. The levels of IL-8 were compared with those detected by stimulating the HT29 with the same amounts of LPS in the absence of serum and the presence of human breast milk (1.7%). In some experiments, the anti-CD14 specific mAb MY4 (20 μg/ml), which binds to the same epitope as LPS on the sCD14 molecule, was used to block the stimulation mediated by the human milk or serum.

As shown in figure 2, neither LPS alone nor human serum or human milk alone were able to induce significant levels of IL-8. However LPS was capable of inducing substantial amounts of IL-8 in the presence of either human serum or human milk. This effect was abrogated by CD14-specific monoclonal antibodies MY4, but not by an irrelevant monoclonal antibody (IgG2b, MOPC-141). Similar results were obtained with the other intestinal epithelial cell line SW620 and with differenciated HT29 cells.

Test 3: mmsCD14 quantitative determination in human milk

Concentration of mmsCD14 in human milk was determined by a human CD14-specific ELISA test (IBL, Germany). Samples taken in the first days of lactation had a higher amount of sCD14 than the overall average: 75.4± 19.1μg/ml

compared to 52.9 ± 24.0µg/ml. This is thought to be at least partially due to leakage of serum sCD14 into the colostrum.

<u>Test 4:</u> Functional effect of milk-derived sCD14

In vitro stimulation studies of astrocytoma (U373) cell lines with several dose of lipopolysaccharides, 10pg/ml to 5pg/ml, in the presence and/or absence of human or bovine milk, human or foetal calf serum, and anti-CD14 antibodies were carried out.

The human astrocytoma cell line U373 (CD14 negative) was stimulated with varying concentrations of lipopolysaccharides, 1pg/ml to 10ng/ml, in the presence or absence of human AB serum (10% or 1%) and soluble recombinant human CD14 (3 ug/ml). After 48h culture at 37°C, culture supernatants were collected and tested for IL-6 release by ELISA. The levels of IL-6 were compared with those detected by stimulating the U373 with the same amounts of lipopolysaccharides in the absence of serum and the presence of 1% human milk. In some experiments, the anti-CD14 specific mAbs MEM-18 and MY4 (15ug/ml), which bind to the same epitope as lipopolysaccharides on the sCD14 molecule, was used to block stimulation mediated by human milk or serum.

Neither lipopolysaccharides alone nor 0.5% or 1% human milk alone are able to induce significant levels of IL-6. However, lipopolysaccharides were capable of inducing substantial amounts of IL-6 in the presence of mature human milk (0.5 and 1%). This effect was abrogated by two CD14-specific monoclonal, antibodies , MEM-18 and MY4, but not by an irrelevant monoclonal antibody (iMab MOPC-21). MEM-18 and MY4 were also able to block the release of IL-6 induced by lipopolysaccharides in the presence of 10 AB human serum, albeit partially, suggesting that other mechanisms of IL-6 release independent of CD14 may operate in human serum but not in mature milk.

In conclusion, mature milk-derived sCD14 is biologically active and capable of mediating cell activation by lipopolysaccharides. Differences in the biological activities are observed between serum sCD14 and mature milk-derived sCD14.

In addition, the contribution of mmsCD14 on the stimulation of intestinal epithelial cells by Gram-negative non-pathogenic bacteria was tested. HT29 cells were stimulated with varying amounts of *E. Coli* (1×10^3 to 5×10^6 CFU per ml) in the presence or absence mature human breast milk (1.7%). After 24h culture at 37°C, culture supernatants were collected and tested for IL-8 release by ELISA. The levels of IL-8 were compared with those detected by stimulating the HT29 with the same amounts of LPS in the absence of milk and the presence of human AB serum (10%).

As shown in figure 3, *E. Coli* alone was unable to induce significant levels of IL-8. However Incubation of E.Coli in the presence of either human serum or human milk was capable of inducing substantial amounts of IL-8. This effect was abrogated by CD14-specific monoclonal antibodies MY4, but not by the irrelevant antibody (IgG2b).

<u>Test 5:</u> **Production of mmsCD14 from natural sources.**

Purification of mmsCD14 with anti-sCD14 monoclonal antibodies, production of anti-mmsCD14 polyclonal antibody and large scale purification immunoaffiniity chromatography and ion exchange chromatography.

a. <u>Immunoaffinity chromatography for purification of sCD14 from mature milk (bovine, buffalo, goat or sheep)</u>

Small scale purification of mmsCD14 was performed by immunoaffinity chromatography using a purified anti-sCD14 monoclonal antibody, MY4 (Coulter Immunotech, USA). Briefly, a diluted mature milk sample was applied to an anti-CD14-Sepharose 4B matrix. After washing, the column was eluted with 100 mM Glycine.HCl, pH 2.5. Fractions are collected and neutralized. The mmsCD14 content of each fraction was determined by an anti-sCD14 ELISA test (IBL, Hamburg, Germany), and the purity was analysed by SDS-PAGE and silver staining. The selected fractions were pooled and kept in –20°C until further use.

Polyclonal antibodies against mmsCD14 were obtained by immunisation of rabbits with the antigen-adjuvant mixture. Briefly, an emulsion composed of 0.5

mg/ml purified mmsCD14 and 2 ml complete Freund adjuvant (Sigma, St Louis, MO) with insoluble Mycobacterium tuberculosis bacilli was injected into multiple intamuscular sites. The animal was bled 14 days following the first immunisation. Booster immunisation using incomplete Freund adjuvant (Sigma) was performed 6 weeks after priming immunisation and with intervals of 2-3 weeks thereafter. Bleedings were performed 10-14 days after immunisation. The antibody titers were performed by ELISA.

Small scale purification of sCD14 from milk was performed by immunoaffinity chromatography using the purified polyclonal antiserum.

Example 1

Formula for low-birth-weight infants, in powder form was prepared.

The formula has the composition (per 100 g of powder) which is described in the table I below.

Table I
[ALPREM NA071]

Nutrient	Unit	Amount
Total fat	g	24
Total protein	g	14.4
Total carbohydrates	g	55.9
mmsCD14	mg	20
Sodium	mg	180
Potassium	mg	530
Chloride	mg	280
Calcium	mg	490
Phosphorus	mg	320
Magnesium	mg	54
Manganese	µg	34
Vitamin A	IU	1500
Vitamin D	IU	490
Vitamin E	IU	9.8

Vitamin K_1	µg	59
Vitamin C	mg	79
Vitamin B_1	mg	0.29
Vitamin B_2	mg	0.66
Niacin	mg	4.9
Vitamin B_6	mg	0.37
Folic acid	µg	290
Pantothenic acid	mg	2.2
Vitamin B_{12}	µg	1.1
Biotin	µg	11
Choline	mg	37
Nutrient	**Unit**	**Amount**
Inositol	mg	22
Taurine	mg	39
Carnitine	mg	7.9
Iron	mg	7.4
Iodine	µg	49
Copper	mg	0.44
Zinc	mg	3.7

The formula was reconstituted by mixing 142g of powder to 900mL of water to give 1L of ready-to-drink preparation. The composition given above can vary to accommodate for local directives concerning the amounts of specific ingredients. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in an adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 2

Starter formula for infants (from birth to 4-5 months), in powder form was prepared.

The formula has the composition (per 100 g of powder) which is described in the table II below.

Table II

Nutrient	Unit	Amount
Total fat	g	25.8
Total protein	g	11.5
Total carbohydrates	g	57.8
mmsCD14	mg	20
Sodium	mg	120
Potassium	mg	460
Chloride	mg	360
Calcium	mg	320
Nutrient	**Unit**	**Amount**
Phosphorus	mg	160
Magnesium	mg	35
Manganese	µg	36
Vitamin A	IU	1500
Vitamin D	IU	310
Vitamin E	IU	6.1
Vitamin K_1	µg	42
Vitamin C	mg	41
Vitamin B_1	mg	0.31
Vitamin B_2	mg	0.69
Niacin	mg	3.8
Vitamin B_6	mg	0.38
Folic acid	µg	46
Pantothenic acid	mg	2.3
Vitamin B_{12}	µg	1.1
Biotin	µg	11
Choline	mg	38
Inositol	mg	23
Taurine	mg	41
Carnitine	mg	8.2
Iron	mg	6.1
Iodine	µg	25
Copper	mg	0.31

Zinc	mg	3.8

The formula was reconstituted by mixing 132g of powder to 900mL of water to give 1L of ready-to-drink preparation. The composition given above can vary to accommodate for local directives concerning the amounts of specific ingredients. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 3

A formula for infants, from 5 months of age, is prepared.

The formula has the composition (per liter of ready to use preparation) which is described in the table III below.

Table III

Nutrient	**Unit**	**Amount**
Total fat	g	29.4
Total protein	g	22.4
Total carbohydrates	g	78.9
mmsCD14	mg	25
Sodium	mg	320
Potassium	mg	1060
Chloride	mg	760
Phosphorus	mg	680
Calcium	mg	820
Magnesium	mg	73
Manganese	µg	41
Vitamin A	IU	2700
Vitamin D	IU	600
Vitamin E	IU	8
Vitamin K_1	µg	30
Vitamin C	mg	67

Vitamin B_1	mg	1
Vitamin B_2	mg	1.6
Niacin	mg	18
Vitamin B_6	mg	1.3
Folic acid	µg	200
Pantothenic acid	mg	4.7
Vitamin B_{12}	µg	1.3
Biotin	µg	23
Choline	mg	67
Nutrient	**Unit**	**Amount**
Inositol	mg	34
Iron	mg	11
Iodine	µg	140
Copper	mg	0.8
Zinc	mg	8

The composition given above can be varied to accommodate for local directives concerning the amounts of specific ingredients. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in an adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 4

A formula for infants, from 5 months of age, containing partly hydrolyzed protein for low allergenicity, in powder form was prepared. The formula has the composition (per 100g of powder) which is described in the followed table IV.

Table IV

Nutrient	Unit	Amount
Total fat	g	22
Total protein	g	15.2
Total carbohydrates	g	57
mmsCD14	Mg	20

Sodium	Mg	170
Potassium	Mg	580
Chloride	Mg	340
Calcium	Mg	540
Phosphorus	Mg	230
Magnesium	Mg	39
Manganese	μg	29
Vitamin A	IU	1900
Vitamin D	IU	440
Vitamin E	IU	5.8
Nutrient	**Unit**	**Amount**
Vitamin K_1	μg	22
Vitamin C	Mg	49
Vitamin B_1	Mg	0.73
Vitamin B_2	Mg	1.2
Niacin	Mg	13
Vitamin B_6	Mg	0.97
Folic acid	μg	150
Pantothenic acid	Mg	3.4
Vitamin B_{12}	μg	0.97
Biotin	μg	17
Choline	Mg	49
Inositol	Mg	24
Iron	Mg	8.3
Iodine	μg	100
Copper	Mg	0.58
Zinc	Mg	5.8

The formula was reconstituted by mixing 138g of powder to 900mL of water to give 1L of ready-to-drink preparation. The composition given above can be varied to accommodate for local directives concerning the amounts of specific ingredients permitted. Other trace elements (e.g. selenium, chromium, molybdenum, fluoride) may be added in an adequate amount according to age of the intended consumer. Nucleosides and/or nucleotides can also be present.

Example 5

A formula that can be administered to an infant suffering from bovine milk allergy, comprising ultrafiltered/microfiltered extensively hydrolyzed protein, in powder form was prepared.

The formula had the composition (per 100 g of powder) which is described in the following table V.

Table V

Nutrient	Unit	Amount
Total fat	g	24
Total protein	g	16.5
Total carbohydrates	g	52
mmsCD14	mg	20
Sodium	mg	290
Potassium	mg	600
Chloride	mg	500
Calcium	mg	400
Phosphorus	mg	250
Magnesium	mg	60
Manganese	µg	337
Vitamin A	IU	1200
Vitamin D	IU	290
Vitamin E	IU	5.8
Vitamin K_1	µg	26
Vitamin C	mg	39
Vitamin B_1	mg	0.29
Vitamin B_2	mg	0.63
Niacin	mg	3.6
Vitamin B_6	mg	0.34
Folic acid	µg	43
Pantothenic acid	mg	2.2
Vitamin B_{12}	µg	0.96

Biotin	µg	11
Choline	mg	58
Inositol	mg	29
Taurine	Mg	39
Carnitine	Mg	14
Iron	Mg	7.2
Iodine	µg	39
Copper	Mg	0.39
Zinc	Mg	3.4
Nutrient	**Unit**	**Amount**
Chromium	µg	14
Molybdenum	µg	39
Fluoride	µg	140

The formula was reconstituted by mixing 150g of powder to 900ml of water to give 1l of a ready-to-drink preparation. The composition can be varied to accommodate local directives concerning the amounts of specific ingredients permitted. Other trace elements (e.g. selenium) may be added in an adequate amount according to the age of the intended consumer. Nucleosides and/or nucleotides can also be present.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

Claims

1. An isolated protein having no O-glyclosylation and at least 70% homology of amino acid sequence with human serum CD14.

2. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of human serum CD14.

3. A protein according to claim 1 wherein its amino acid sequence is at least about 95% homologous with the amino acid sequence of bovine or buffalo CD14.

4. A protein according to any preceding claim wherein the protein has a plurality of N- glycosylation sites.

5. A protein according to claim 4 which comprises from about 3 to about 5 N- glyclosylation sites.

6. A protein according to any preceding claim wherein the presence of the protein is not revealed in a Western blot by the known commercially available anti-CD14 monoclonal antibody MY4.

7. A protein according to any preceding claim isolated from mature human, bovine or buffalo milk.

8. A method of production of the protein which comprises isolating it from mature milk.

9. A composition which comprises a protein according to any one of claims 1 to 7 excluding mature milk.

10. A composition according to claim 9 which comprises a physiologically acceptable carrier, adjuvant or diluent.

11. A composition according to claim 9 or 10 which comprises a casein fraction and milk fat.

12. A composition according to any one of claims 9 to 11 which comprises a lipopolysaccharide binding protein (LBP), decay accelerating factor (DAF, CD55), bactericidal permeability increasing factor (BPI) or a mixture thereof.

13. A composition according to any one of claims 9 to 12 in the form of an infant formula or enteral composition.

14. A composition according to any one of claims 9 to 13 which comprises at least 25μg/ml of a protein according to any one of claims 1 to 7.

15. A method of production of the composition which comprises adding a protein according to any one of claims 1 to 7.

16. Use of a CD14 variant or fragment that retains the bioactivity of CD14 in the manufacture of a nutritional product or medicament for the treatment or prevention of a GI tract disorder.

17. Use according to claim 16 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonotal sepsis, infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

18. A method of treatment or prevention of a GI tract disorder which comprises administering an effective amount of a CD14 variant or fragment thereof which retains the bioactivity of CD14.

19. A method of treatment according to claim 18 wherein the GI tract disorder is selected from the group which comprises inflammatory bowel disease, Chrone's disease, ulcerative cholitis, coeliac disease, intestinal bacterial overgrowth, chronic hepatitis, necrotising enterocolitis, neonotal sepsis,

infectious diarrhoea, disbalance of the intestinal microflora, allergic reactions to food and bacterial translocation from the gut to other compartments of the body.

Figure 1

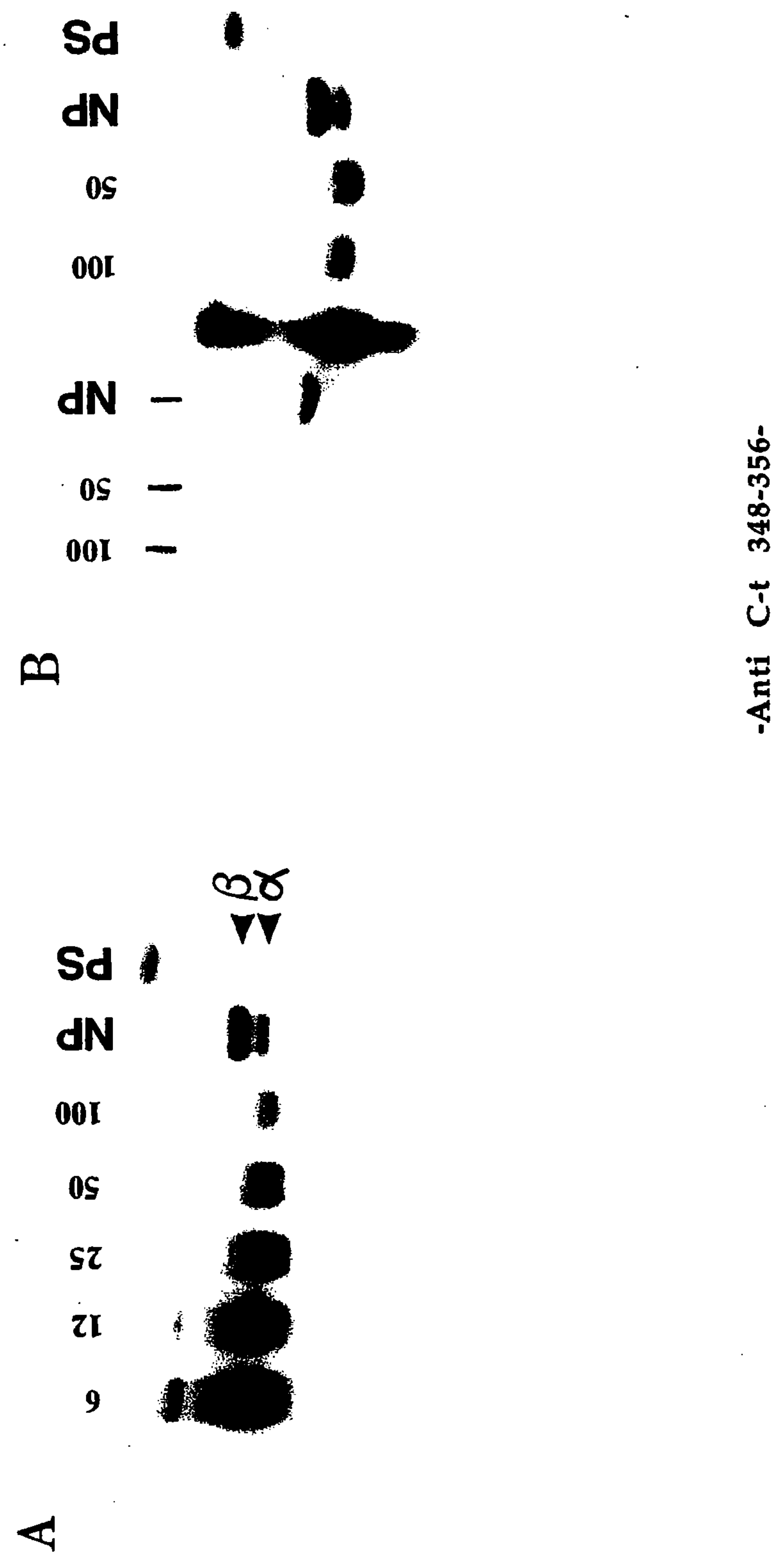
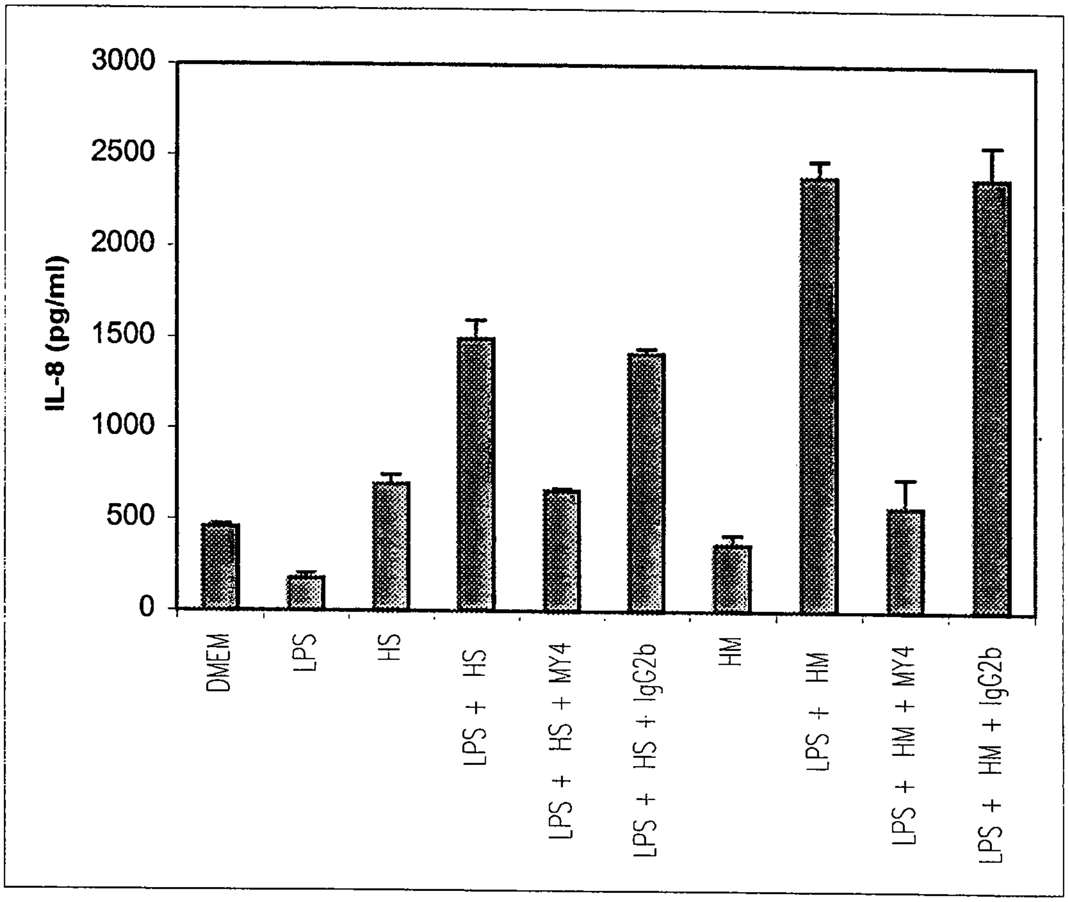


Figure 2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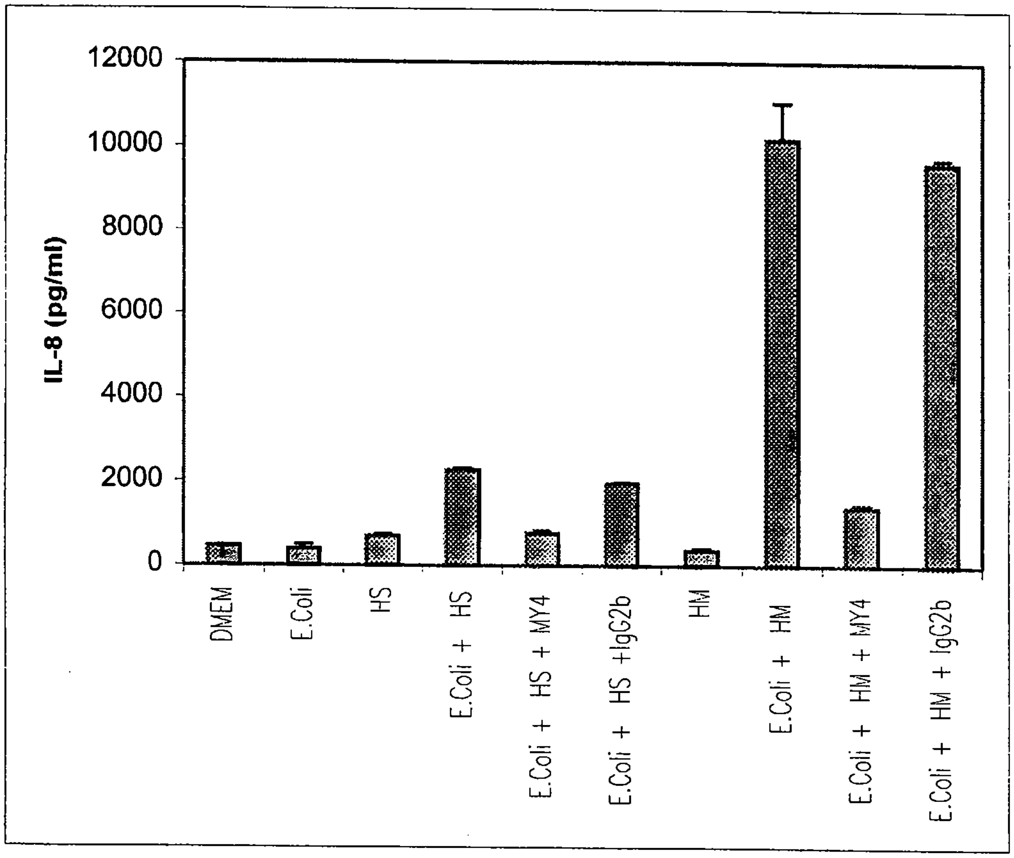
2500
2000
1500
1000
500
0
DMEM
LPS
HS
LPS + HS
LPS + HS + MY4
LPS + HS + IgG2b
HM
LPS + HM
LPS + HM + MY4
LPS + HM + IgG2b

Figure 3



IL-8 (pg/ml)
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10000
8000
6000
4000
2000
0
DMEM
E.Coli
HS
E.Coli + HS
E.Coli + HS + MY4
E.Coli + HS +IgG2b
HM
E.Coli + HM
E.Coli + HM + MY4
E.Coli + HM + IgG2b